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Molecular Characterization and PCR Diagnosis of Thailand Deletion of α-Globin Gene Cluster

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Thailand deletion of α-Thalassemia (thal) 1 involves the ζ2-, φζ1-, α2-, α1-, and θ1-globin genes. In Southeast Asians and Taiwanese, this mutation is the second most common long-segment deletion of two α-globin genes, after the Southeast Asian deletion. To define the Thailand deletion breakpoints, we used polymerase chain reaction (PCR) to amplify the normal-sequence DNA fragments across the breakpoints. The amplified products were sequenced directly or after cloning into pGem®-3Z or pCR®2.1 vectors. Comparison of the normal and mutant sequences revealed that the 5′ breakpoint lies between nucleotides 1,269 and 1,290 upstream of the initiator codon adenine of the ζ2-globin gene, and the 3′ breakpoint lies between nucleotides 29,387 and 29,408 downstream of it. A total of 30,677 nucleotides were deleted. Both breakpoints mentioned above lie within the Alu repetitive sequences and an extensive sequence homology is present around the two breakpoints. These findings suggest that homologous recombination is the mechanism by which the deletion occurs. Based on our data, we used three oligonucleotide primers to amplify the regions across the deletion and its corresponding normal sequence. The feasibility of PCR diagnosis was confirmed in 20 carriers with this deletion. Am. J. Hematol. 57:124–130, 1998.

Key words: α-thalassemia 1, Thailand deletion, molecular characterization, polymerase chain reaction diagnosis

INTRODUCTION

The α-globin gene cluster has been mapped to the terminal portion of the short arm of chromosome 16. The arrangement of the cluster is 5′-ζ2-φζ1-φα2-φα1-α2-α1-θ1-3′, and the whole cluster spans about 50 kb [1–3]. A normal α-globin gene cluster is designated as αα/. The cluster is prone to long-segment deletions involving both α-globin genes, which results in α-thalassemia (thal) 1 (designated as --/). More than ten different α-thal 1 deletions have been reported worldwide, with the Mediterranean deletion (--MED), the Southeast Asian deletion (--SEA), and a 20.5-kb deletion involving an Alu repeat and α1-globin gene [$-(\alpha)^{20.5}$] being the most common [4–16].

The Thailand deletion (--THAI) reportedly involves a more extensive deletion than the three more common ones mentioned above [4,5,7,9]. The ζ2-globin gene, which expresses its function in the embryonic stage, is also deleted in --THAI. Embryos with homozygous --THAI deletion may experience early spontaneous abortion because the normal embryonic hemoglobins (Hb) [Hb Gower 1 (ζ2ε2) and Hb Portland (ζ2γ2)] are absent. Compound heterozygotes for --THAI and --SEA or other α-thal 1 determinants lead to Hb Bart's hydrops fetalis [12]. The precise breakpoints of --MED, --SEA, and $-(\alpha)^{20.5}$ have been well characterized [7,17]. The regions deleted by these defects are around 20 kb in length. Both homologous recombination involving the Alu family repeat and illegitimate recombination have been proposed as the mechanisms leading to these deletions [17]. In Taiwan, 4.5% of the residents are carriers

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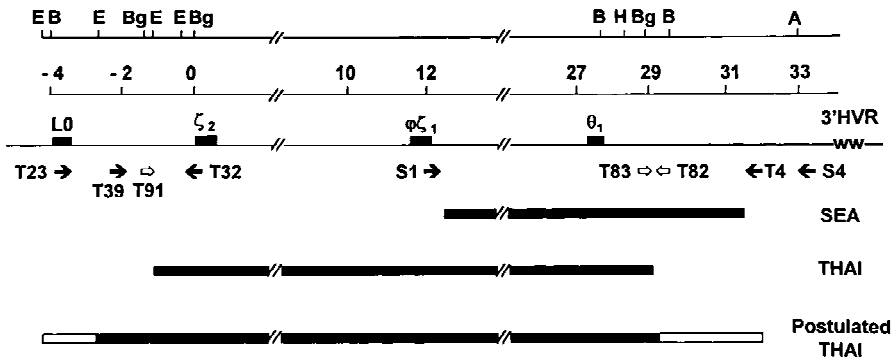

Fig. 1. Deletion extent of --SEA; postulated and characterized extents of --THAI. Coordinates are given in kb: 0 represents the initiator codon adenine of the ζ2-globin gene. B = BamHI, Bg = Bag II, E = EcoRI, H = HindIII, A = AccI. Black bars indicate the deleted region and white bars indicate the segments within which the breakpoints are supposed to lie. Black arrows indicate the primers used for DNA amplification and sequencing. White arrows indicate the primers used for DNA amplification across the --THAI deletion and its corresponding normal sequence.

of α-thal 1 [18]. Most (96%) result from --SEA and another 3% from --THAI [12]. In the aboriginal Taiwan people, --THAI is especially common. Four percent of the Ami are α-thal 1 carriers and 75% due to --THAI [19]. --THAI is also a common cause of α-thal 1 in Southeast Asians [5,9]. The restriction map around the --THAI determinant has been described; however, the exact breakpoints have not been reported. According to previous studies, the 5′ breakpoint is between the L0 fragment and the ζ2-globin gene and the 3′ breakpoint is between the θ1-globin gene and a 3′ hypervariable region (3′HVR) [9,20]. Molecular characterization of the exact breakpoints will shed light on the mechanism leading to this deletion. Thus, rapid diagnosis can be accomplished using polymerase chain reaction (PCR)-based techniques [21–23].

MATERIALS AND METHODS

The initiator codon adenine of ζ2-globin gene is designated as at coordinate 0. The θ1-globin gene is located between coordinates 27 and 28 (1 unit is 1 kb), the 3′HVR is at about coordinate 34 to 35, and the L0 fragment is at coordinate −4 [9,20] (Fig. 1). The deletion of --SEA occurs between coordinates 12.5 and 31.5 [17,24]. The deletion of --THAI has been reported to extend from the 3′ flanking region of the L0 fragment (between coordinates −4 and −2.5) to a region between the θ1-globin gene and the 3′HVR region [9] (between coordinates 29.5 and 32) (Fig. 1).

Using DNA from a normal subject, the sequences from the L0 fragment (coordinate −4) to the ζ2-globin gene (coordinate 0) were determined first. Using DNA from a --SEA carrier, the sequence between coordinates 31.5 and 33 was determined. Afterwards, a junction fragment across the --THAI breakpoints was amplified and sequenced. Comparison of the differences between the normal and mutant sequences and between our data and the recently published sequences (GenBank accession numbers Z84721 and Z69706) showed the exact breakpoints [25].

Sequencing of the L0 Fragment

The 0.5-kb EcoRI-BamHI L0 fragment was isolated from pBam 6.8 and then subcloned onto the EcoRI and BamHI sites of pGEM®-3Z [9] (Promega, Madison, WI). The entire L0 fragment was sequenced using M13 forward and reverse sequencing primers. Elution of the PCR product from the agarose gel, ligation of the insert DNA with the vector, *Escherichia coli* transformation, colony selection, preparation of the plasmid DNA, and restriction enzyme digestion were then conducted following standard methods or the instructions recommended by the respective manufacturers. Direct DNA cycle sequencing was performed using dye-labeled terminators (ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit, With AmpliTaq® DNA Polymerase FS), and an ABI PRISM 377 DNA Sequencer (Perkin-Elmer Cetus, Norwalk, CT).

Sequencing of the Region Between the L0 Fragment and the ζ2-Globin Gene

Two primers, T23 and T32, were used to amplify this DNA fragment. The 5′-end primer, T23 (5′-gtaaggccagagtgggtgggcac-3′), was designed according to the sequence at the 3′ end of the L0 fragment. The 3′-end primer, T32 (5′-aagcaggaggaggcagcaggtcc-3′) was designed according to the 5′-flanking sequence of the ζ2-globin gene (GenBank, accession numbers Z00182 and Z00181). We used the Expanded Long Template PCR System (Boehringer Mannheim GmbH, Mannheim, Germany) for DNA amplification and expected to obtain a 3–4-kb fragment. Conditions for PCR were 30 cycles each at 94°C for 30 sec, and 68°C for 5 min (DNA Thermocycler, Perkin Elmer-Cetus, Model 9600). The fragment was sequenced from both ends using T23, T32 and other additional primers (sequences not shown) as appropriate. When we countered regions that were diffi-

cult to sequence directly, such as poly-A or poly-T tract, the remaining DNA fragment was PCR-amplified and then cloned into pCR®2.1 (Original TA Cloning Kit, Invitrogen Corporation, San Diego, CA) following standard methods and the instructions recommended by the manufacturers. The cloned fragment was sequenced from both ends with M13 forward and reverse primers.

Sequencing of the Region Between Coordinates 31.5 and 33

DNA from a--SEA carrier was used as a PCR template. Two primers, S1 (5′-gtgttctcagtattggagggaa-3′) and S4 (5′-ccttacctgcacctttttcctgcag-3′) [17,20,21,24] were used and expected to amplify a 1–2-kb fragment. The complete sequence of the fragment was determined using primer S1, S4, and additional ones (sequences not shown) as appropriate. The--SEA breakpoint was expected to be seen near the S1 primer [17].

Sequencing Across the THAI Deletion

Several primers were designed according to our sequenced data for the area between coordinates 31.5 and 33. Each time, we paired one of these primers with T23 in a long-distance PCR reaction, expecting to obtain a PCR product. Once a PCR product was obtained, we did preliminary sequencing from both ends to determine if the fragment did bridge the THAI deletion. A cloning and sequencing strategy would be used if we encountered a poly-A or poly-T tract that was difficult to sequence directly. A comparison of the sequence from this fragment with the normal sequence would disclose the breakpoints of--THAI.

Gap-PCR Diagnosis of the THAI Deletion

Three primers, T91, T83, and T82, were synthesized for PCR diagnosis of this deletion. T91 (5′-taaaccacccaattttaaatgggc-3′, nucleotide 1,459 to 1,483 upstream of the ζ2-globin gene initiator adenine, corresponding to nucleotide 12,290 to 12,314 in Z84721) is in the 5′ flanking region of the ζ2-globin gene. T83 (5′-ggtattctcaaggtgacactgagg-3′, nucleotide 29,189 to 29,212 downstream of the ζ2-globin gene initiator adenine, corresponding to nucleotide 42,962 to 42,985 in Z84721 or 7 to 30 in Z69706) and T82 (5′-ataacctttatctgccacatgtagc-3′, nucleotide 29,728 to 29,752 downstream of the ζ2-globin gene initiator adenine, corresponding to nucleotide 546 to 570 in Z69706) are in the 3′ flanking region of the θ1-globin gene. T82 in combination with T91 was expected to amplify a 560-bp fragment in carriers of--THAI while T82 in combination with T83 was expected to amplify a 564-bp fragment in normal subjects or carriers of--THAI. For these PCRs, 1 μl (0.5 μg) of genomic DNA, 1 μl (10 pmol) each of the primers, 2.5 μl of 10 × PCR buffer, and 18.3 μl of pure water were mixed and heated at 95°C for 8–10 min for denaturation; then 1 U (0.2 μl) of Taq polymerase (Perkin Elmer) and 2 μl of dNTPs were added. Thirty PCR cycles were executed in a programmable thermocycler (Model 4800, Perkin Elmer-Cetus) under the following

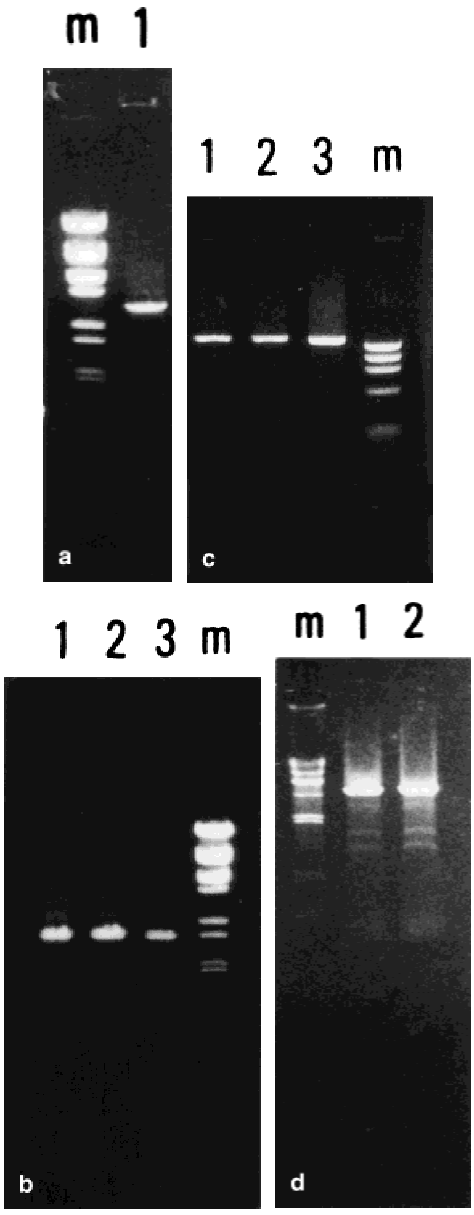

Fig. 2. a: Ethidium bromide-stained photograph of a 3.5-kb fragment amplified with T23 and T32 from a normal subject TK (lane 1). m = DNA size marker of λ-HindIII. b: Ethidium bromide-stained photograph of ~2-kb fragment amplified with T39 and T32 from TK (lane 1) and two other subjects (lanes 2 and 3). m = DNA size marker of λ-HindIII. c: Ethidium bromide-stained photograph of a 1.5-kb fragment amplified with S1 and S4 from three carriers with an --SEA determinant (lanes 1–3). m = DNA size marker of ϕX174-HaeIII. d: Ethidium bromide-stained photograph of a 4.8-kb fragment amplified with T23 and T4 from two carriers with a --THAI determinant (lanes 1 and 2). m = DNA size marker of λ-HindIII.

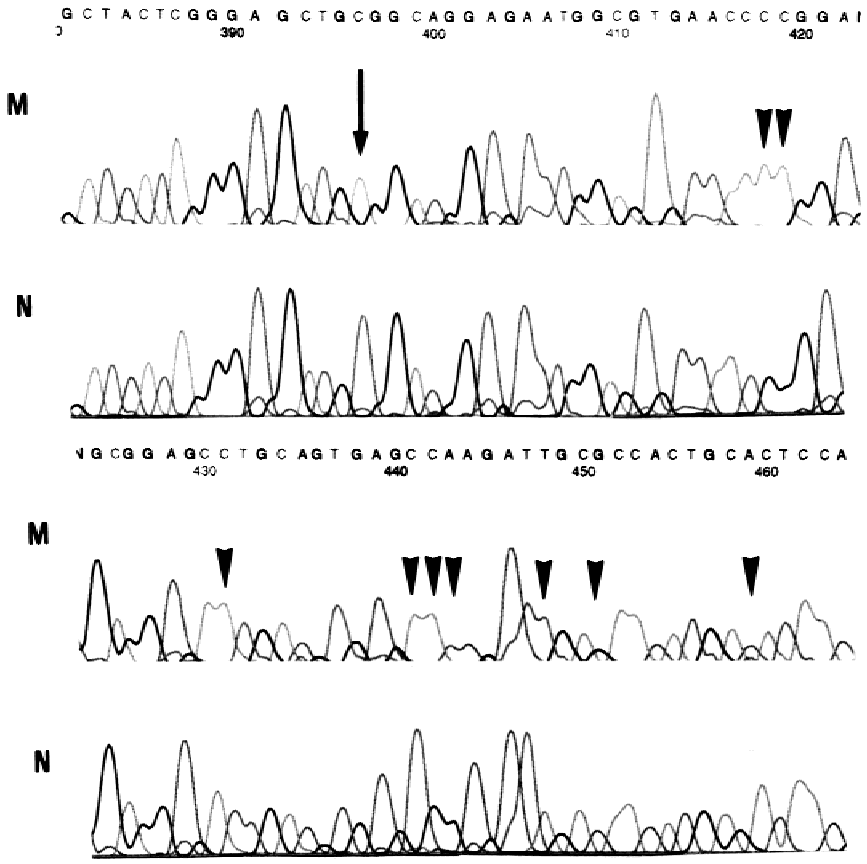

Fig. 3. DNA sequencing across the --THAI breakpoints. M = mutant sequence, N = normal sequence. The arrow indicates the first nucleotide change. The arrowheads indicate the nucleotides specific for the 3′ normal sequences.

conditions: 94°C, 30 sec for denaturation, 62°C, 30 sec for annealing, and 72°C, 1 min for extension. The final extension cycle was 7 min. The PCR products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide. Ten normal subjects were studied using T91 + T82 and T83 + T82. Twenty --THAI carriers were studied using T91 + T82. From one --THAI carrier, PCR products amplified with T91 + T82 were purified and sequenced directly to confirm the presence of --THAI breakpoints.

RESULTS

The L0 fragment had an identical sequence to that recently reported by Flint et al. in the telomeric region of the short arm of chromosome 16 [25]. T23 and T32 amplified a 3.5-kb fragment (Fig. 2a). From the 5′-end, sequencing proceeded smoothly for about 1.5-kb, using 5 additional primers until a poly-A tract was encountered, while at the 3′-end, only a short sequence was defined before we encountered a poly-T tract. This remaining ~2-kb region was amplified with primers T39 (5′-cctgaccaacatggagaaaccccgac-3′) and T32 (Fig. 2b). The sequence was obtained after cloning into pCR®2.1. A comparison with the data by Flint et al. revealed 4 differences [25]: two involved a poly-A tract (at nucleotides 11,451 and 11,615 of Z84721, respectively), and two involved an adenine→guanine change (at nucleotides 11,487 and 12,320 of Z84721, respectively).

S1 and S4 amplified a 1.5-kb fragment (Fig. 2c). As expected, the --SEA breakpoint [17] was seen near the S1 primer, indicating that we had obtained the correct sequence.

To obtain a fragment across the THAI breakpoint, only one primer T4 (5′-gagacgatgcttgctttgtcacccatgctggag-3′) in combination with T23 amplified a 4.8-kb DNA fragment (Fig. 2d). Preliminary sequencing from both ends confirmed that this fragment was across the breakpoint. Direct sequencing from the 3′ end using T4 proceeded for about 0.6-kb before encountering a poly-T tract. From the 5′ end, DNA sequencing proceeded smoothly for 2.6-kb using 7 additional primers, with the sequencing of the first 2.5-kb being identical to that of a normal Chinese, and then a gradual divergence from our previously obtained data was noted (Fig. 3). Figure 4 shows the sequence divergences among the 5′ normal sequence around coordinate −1, the sequence across the --THAI deletion, and the 3′ normal sequence around coordinate 29 (GenBank accession numbers Z84721 and Z69706). It is clear that the 5′ breakpoint was between nucleotides 12,483 and 12,503 in Z84721, and the 3′ breakpoint was between nucleotides 204 and 225 in Z69706. Using the

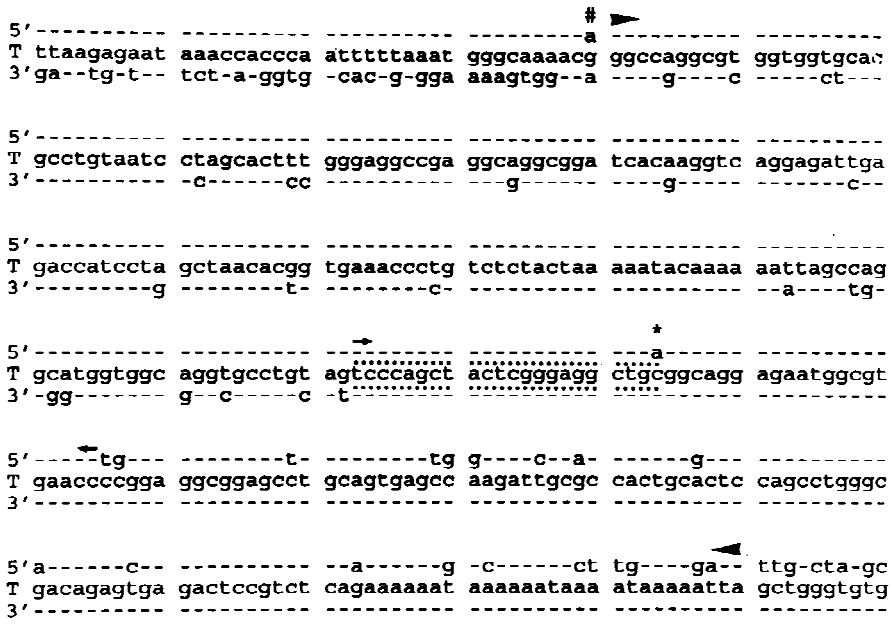

Fig. 4. Comparison of the 5′ normal (top), 3′ normal (bottom), and the mutant sequence (middle) across --THAI deletion. The ---- symbols indicate the same nucleotide as the mutant sequence. The * indicates the first nucleotide change in the 5′ normal sequence to the 3′ normal sequence. The dotted stretch of nucleotides indicate the region within which homologous recombination might have occurred (adenine to cytosine). # = polymorphic guanine/adenine. The arrowheads indicate a 21-nucleotide stretch of 100% sequence homology on both sides of the divergent adenine/cytosine. The arrows indicate the region with 87% sequence homology between the 5′ and 3′ normal sequences.

aforementioned designation system, the 5′ breakpoint lies between nucleotides 1,269 and 1,290 upstream of the initiator codon adenine of the ζ2-globin gene, and the 3′ breakpoint lies between nucleotides 29,387 and 29,408 downstream of it. A total of 30,677 nucleotides were deleted. The 5′ breakpoint lies within an Alu-Y repetitive sequence, the 3′ breakpoint lies within an Alu repetitive sequence, and an extensive sequence homology is present around each of the two breakpoints [25].

In PCR diagnosis, T82 and T91 amplified an expected 560-bp fragment from 20 --THAI carriers and did not amplify any fragments from normal subjects. T82 and T83 amplified another 564-bp fragment from normal subjects (Fig. 5). Direct DNA sequencing of PCR products generated with T82 and T91 confirmed that the --THAI deletion breakpoint did exist in the fragment (Fig. 6).

We have completely sequenced 4,500 nucleotides in the α-globin gene cluster. In addition to the four sequence differences noted in the region between coordinates 0 and −4, a fifth involved an insertion of a guanine at nucleotide 252 of Z69706, just 28 nucleotides 3′ to the divergent a/c. Both the 5′ and 3′ normal sequences in our data had an extra guanine at this site (Fig. 4). DNA polymorphism between various ethnic groups may be the most important factor in accounting for these differences.

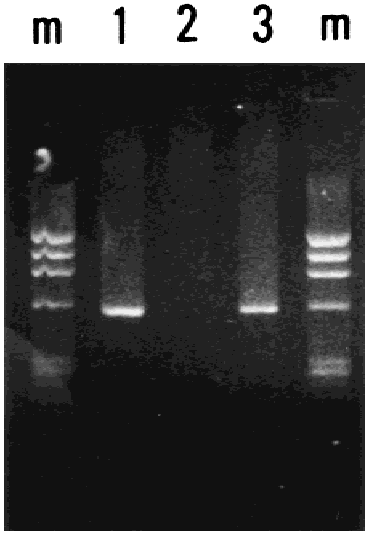

Fig. 5. Ethidium bromide-stained photograph of gap-PCR for rapid diagnosis of --THAI. m = DNA size marker of ϕX174-HaeIII. Lane 1 = T83 + T82, normal subject, a DNA fragment of 564 bp is seen. Lane 2 = T91 + T82, normal subject, no DNA fragment could be amplified. Lane 3 = T91 + T82, a carrier of --THAI, a DNA fragment of 560 bp is seen.

DISCUSSION

We have defined the breakpoints of --THAI by comparing the mutant sequence across the breakpoint and the normal sequence in the α-globin gene cluster. A total of 30,451 nucleotides are deleted. The breakpoints differed from those proposed in previous studies (Fig. 1). The deleted lengths reported in previous studies were at least 2 kb longer than those we observed. Comparison of the mutant and normal sequences revealed extensive sequence homologies. The 5′ normal sequence occurs within an Alu-Y repetitive sequence, and the 3′ normal sequence also occurs within an Alu repetitive sequence [25]. With the divergent nucleotide 42,955 a/c as a center, there is a 21-nucleotide stretch of 100% sequence homology between the 5′ and 3′ normal sequences on each flanking side. In total, there is an 87% sequence

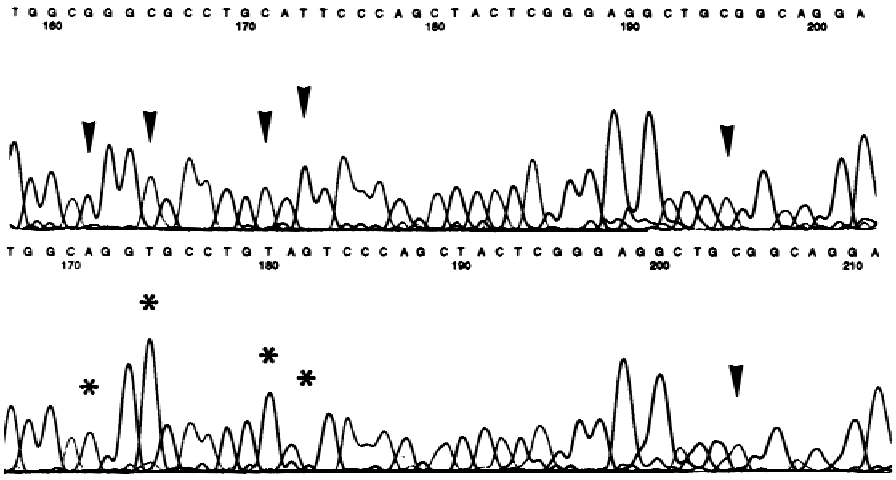

Fig. 6. Direct DNA sequencing of PCR products obtained using primers T83 and T82 (for sequence at coordinate 29, top), and using primers T91 and T82 (for sequences at coordinates −1 and 29, bottom). The arrowheads indicate the nucleotides specific at coordinate 29, and the asterisks indicate the nucleotides specific at coordinate −1. The arrowhead (bottom) indicates the site of conversion of adenine to cytosine as shown in Figure 4.

homology between these two regions, starting at the polymorphic g 184 nucleotides upstream of the divergent a/c and ending at the 126th nucleotide downstream of it (Fig. 4). Our results suggest that homologous recombination is the mechanism leading to this long-segment deletion [17].

The α-globin gene cluster is interspersed with Alu-family repeats, which have been reported to be commonly associated with DNA recombination. Another long-segment deletion with a similar mechanism is $(\alpha\alpha)^{RA}$, which results from a simple crossover event between the Alu sequences at coordinate −52 and coordinate +10 [17]. It seems clear that --THAI deletion is also an example of a simple linear chromosomal deletion between two highly homologous Alu repeats in the α-globin gene cluster.

Using primers bridging the deletion region, we were able to obtain consistent results from gap-PCR across the breakpoints. Direct sequencing of the products from gap-PCR confirmed our previous findings. The common long-segment deletions, i.e., $-(\alpha)^{20.5}$, --SEA, and --MED, can be diagnosed using PCR-based methods [21,25]. Our characterization of --THAI will enable us to diagnose carriers of this deletion using PCR. This will be very helpful in the classification of α-thal carriers and in prenatal diagnosis of homozygous α-thal 1. In PCR diagnosis, primers T83 and T82 can amplify a DNA fragment from sequences with --THAI, and primers T91 and T82 can amplify a DNA fragment with this deletion. T91 and T82 did not generate any PCR products from normal subjects, which was consistent with the expected results (Fig. 5). In our PCR experiment, the sizes of DNA fragments from the normal and mutant alleles were almost the same; therefore, simultaneous amplification was not tried. We could design other primers that allow simultaneous amplification of the normal and mutant alleles. This would allow reactions from one pair of primers to be used as controls for the other pair of primers. Because many Alu repeat family sequences were around the two breakpoints, we used direct sequencing on the gap-PCR products to test the specificity of T82 and T91. The presence of the deletion breakpoint in the gap-PCR fragment confirmed our findings and the feasibility of rapid --THAI diagnosis. Characterization of the breakpoints of --THAI will facilitate carrier screening of α-thal and prenatal diagnosis of homozygous α-thal 1 in many parts of the world [26–28].

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